Hormonal Regulation of Cytosolic Free Calcium and Its Functional Consequences: The GH-Cell Model

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Use of clonal strains of prolactin (PRL)- and growth hormone-producing rat pituitary cells has proven informative in elucidating a number of the early biochemical, ionic, and secretory events regulated by the hypothalamic tripeptide, thyrotropin-releasing hormone (TRH). TRH causes biphasic changes in the concentration of cytosolic free calcium ($[Ca^{2+}]_i$) in GH_4C_1 cells and biphasic changes in hormone secretion. Early changes occur on a msecond to second scale and late changes, on a time scale of minutes. Although increases in $[Ca^{2+}]_i$ are essential for enhanced secretion, at least in the case of the rapid initial phase, the TRH-induced increase in $[Ca^{2+}]_i$ is necessary, but not sufficient to enhance secretion. A co-mediator with calcium appears to be diacylglycerol.

The majority of the calcium involved in the early phase of rise in $[Ca^{2+}]_i$ induced by TRH is derived from intracellular sources, while essentially all of the calcium rise observed in the late phase is derived from extracellular calcium entering the cell through both voltage-dependent and voltage-independent conductances. Because TRH causes an elevation of inositol(1,4,5) trisphosphate $[Ins(1,4,5)P_3]$ within seconds, but not mseconds, further studies are required before it can be concluded unequivocally that $Ins(1,4,5)P_3$ is the sole mediator of the rapid phase of rise in $[Ca^{2+}]_i$ induced by TRH in GH-cells. Additional experiments with GH_4C_1 cells have revealed that: *a*) maximal ligand-stimulated activation of protein kinase C requires a rise in $[Ca^{2+}]_i$ as well as diacylglycerol; *b*) the rapid action of TRH on $[Ca^{2+}]_i$ is potentiated by 1,25(OH)$_2$-cholecalciferol by a mechanism that involves enhanced entry of extracellular calcium; *c*) TRH regulates the Na^+/H^+ exchanger in GH-cells to cause cytosolic alkalinization; and finally, *d*) we have recently solubilized the TRH receptor from GH_4C_1 cells in a functionally active form that is coupled to its guanine nucleotide-binding and transducing protein.

Introduction

This paper summarizes the results of experiments performed in my own laboratory on the regulation of cytosolic free calcium $[Ca^{2+}]_i$ and prolactin (PRL) secretion by thyrotropin-releasing hormone (TRH). The model we have investigated extensively uses the so-called GH-cell system, particularly GH_4C_1 and GH_3 cells (1-3). These cells have been used extensively in many laboratories worldwide to study the regulation of the expression of the growth hormone and PRL genes, the mechanisms of action of vasoactive intestinal peptide, somatostatin, epidermal growth factor, platelet-derived growth factor, bombesin, thyroid hormones, glucocorticoids, estradiol, and 1,25(OH)$_2$-cholecalciferol (1,25(OH)$_2$D$_3$) as well as the phorbol ester tumor promoters. Furthermore, a number of studies have used electrophysiological methods to characterize ion channels and cation conductances across the plasma membrane of these cells. In this synopsis I shall not cite any of the many and important contributions from other investigators.

I have chosen to discuss several specific aspects of the roles of $[Ca^{2+}]_i$ and inositol phospholipids in hormone-receptor signaling pathways in GH-cells. A number of generalities can be derived from this cell system, but it is not representative of all protein-secreting cells that use inositol lipids and their hydrolysis products for intracellular signaling. This conclusion does not, however, limit the usefulness of GH-cells but indicates that other models need to be studied in comparable detail.

Early Actions of TRH on Cytosolic Free Calcium

The actions of TRH on GH_4C_1 cells mediated via its specific plasma membrane receptor (4-6) that occur over a time span of several hundred mseconds to several minutes include the following: *a*) increases in $[Ca^{2+}]_i$ that are biphasic, *b*) enhanced inositol lipid

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hydrolysis, c) release of cell calcium into the extracellular environment, d) influx of extracellular calcium, e) enhanced number of calcium-dependent action potentials, f) activation of protein kinase C and other protein kinases, g) altered pattern of protein phosphorylation, h) increased intracellular pH (pH_i), and i) release of stored, previously synthesized PRL that is biphasic.

Three major experimental approaches have been used to examine the early actions of TRH. These are electrophysiological studies, $^{45}Ca^{2+}$ flux analyses, and biochemical investigations of phospholipid turnover and protein phosphorylation. Most investigators agree that a rise in $[Ca^{2+}]_i$ is essential to trigger the PRL release enhanced by TRH; however, there have been uncertainties or differences between laboratories regarding the source or sources of the calcium that contribute to the increase in $[Ca^{2+}]_i$. I shall describe the results of a series of experiments performed largely by Paul Albert, a graduate student in my laboratory, which, coupled with earlier results of Kut-Nie Tan, have lead us to conclude that both the redistribution of intracellular calcium and entry of extracellular calcium play important roles in the biphasic pattern of PRL secretion that is elicited by TRH.

Using Quin-2 to monitor $[Ca^{2+}]_i$, we have identified two phases of change in $[Ca^{2+}]_i$ induced by TRH (7). An acute spike occurs rapidly and decays with a half-time of about 7.5 sec toward the baseline $[Ca^{2+}]_i$. Within about 300 msec after addition of TRH, there is a highly significant increase in $[Ca^{2+}]_i$ that peaks at 1 to 2 sec (8). After the decay of the spike phase, a secondary rise in $[Ca^{2+}]_i$ occurs that reaches a new plateau value for $[Ca^{2+}]_i$ within about 3 min. This secondary plateau is persistent for many minutes in the continued presence of TRH. Thus, the changes in $[Ca^{2+}]_i$ induced by TRH are biphasic.

For the burst phase of increase in $[Ca^{2+}]_i$, the majority (about 80%) is derived from intracellular calcium stores (8). However, the plateau phase of rise in $[Ca^{2+}]_i$ is eliminated by the removal of extracellular calcium (8).

Relationship between the Biphasic Changes in $[Ca^{2+}]_i$ and Prolactin Secretion

Changes in $[Ca^{2+}]_i$ and PRL release were monitored simultaneously in Quin-2-loaded GH_4C_1 cells. After addition of TRH, there is a biphasic change in PRL secretion (8,9). An initial burst of PRL release occurs within the first 1 to 2 min, which is reflected in a 10- to 20-fold increase in the interval rate of secretion. Thereafter, the rate of TRH-enhanced secretion falls to a new sustained rate that is 2- to 3-fold above the control rate. Temporal analysis of changes in $[Ca^{2+}]_i$ and secretion indicates that the spike phase of increase in $[Ca^{2+}]_i$ precedes the burst phase of

increased PRL release by 10 to 20 sec and that the plateau phase of $[Ca^{2+}]_i$ and sustained PRL secretion occur essentially simultaneously (8,9).

To dissect the causative interrelationship between the two phases of TRH action on $[Ca^{2+}]_i$ and secretion, we searched for selective blockers of the burst and plateau phases and found that ionomycin could selectively block the burst phases and that the voltage-gated calcium channel antagonists (nifedipine and verapamil) could depress basal $[Ca^{2+}]_i$ and the plateau phases of the $[Ca^{2+}]_i$ rise and secretion without altering the TRH-induced spike phases. Low concentrations of ionomycin (10–100 nM) cause a spike in $[Ca^{2+}]_i$ in GH_4C_1 cells that resembles closely the spike produced by TRH (8,10). To test whether a redistribution of cellular calcium or influx of extracellular calcium was responsible, we pretreated cells with EGTA before adding ionomycyn. The conditions we used blocked completely K^+-induced influx of extracellular calcium. Chelation of extracellular calcium with EGTA did not prevent the spike in $[Ca^{2+}]_i$ induced by ionomycin. Therefore, the burst in $[Ca^{2+}]_i$ caused by ionomycin is due to release of intracellular calcium. This pattern of change in $[Ca^{2+}]_i$ induced by ionomycin in the presence of EGTA was very similar to the pattern of change in $[Ca^{2+}]_i$ induced by TRH under the same conditions (8).

The similarities of the pattern induced by TRH and ionomycin suggested that both agents might be acting on the same cellular calcium reservoir. Therefore, we determined whether or not TRH could cause a spike in $[Ca^{2+}]_i$ if the peptide were added to cells after ionomycin. TRH did not induce a spike in $[Ca^{2+}]_i$ if it was added 5 min after ionomycin (8); however, the TRH-induced plateau phase of increase in $[Ca^{2+}]_i$ induced by TRH was fully intact after ionomycin pretreatment. Therefore, the TRH-induced plateau in $[Ca^{2+}]_i$ is not dependent on a prior TRH-induced spike in $[Ca^{2+}]_i$. We interpret these results to indicate that ionomycin and TRH act on the same intracellular pool of calcium, and prior treatment with ionomycin depletes that pool rendering it unavailable to the action of TRH.

Having identified the effects of ionomycin on $[Ca^{2+}]_i$, we then examined its actions on basal and TRH-stimulated PRL secretion. Ionomycin alone, despite causing a spike in $[Ca^{2+}]_i$, did not cause a burst in the secretion of PRL (8). Therefore, the spike in $[Ca^{2+}]_i$ alone is not sufficient to cause a burst of secretion. If ionomycin was added to the cells 5 min before TRH, the TRH-induced spike in $[Ca^{2+}]_i$ was blocked, and the TRH-induced burst of PRL secretion was also blocked completely (8). Ionomycin did not block the TRH-induced plateau phase of enhanced secretion. Therefore, a spike in $[Ca^{2+}]_i$ is needed for the burst phase of secretion induced by TRH, but the spike in $[Ca^{2+}]_i$ alone is not sufficient.

We have performed analogous experiments with calcium channel antagonists that block voltage-dependent calcium channels in GH_4C_1 cells (8). Nifedipine and verapamil block that component of the

TRH-induced plateau phases of $[Ca^{2+}]_i$ and enhanced secretion that are due to calcium entry via voltage-dependent calcium channels (about 60% of extracellular calcium enters through these channels); however, nifedipine or verapamil do not block the TRH-induced spike in $[Ca^{2+}]_i$ or the bust in PRL secretion.

Based on knowledge from other laboratories that TRH activates phospholipase C via its receptor and a G-protein, we consider it likely that diacylglycerol (DAG) acts as a co-mediator with calcium in the burst phase of PRL secretion. This conclusion is supported by the results of two kinds of experiments. First, we reconstituted the precise TRH-induced pattern of change in both $[Ca^{2+}]_i$ and PRL secretion using ionomycin to cause the spike in $[Ca^{2+}]_i$ plus the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) to mimic the action of endogenous DAG (9). When secretion was examined, the TRH-induced pattern was also reproduced, essentially identically, by the combination of ionomycin and TPA (9). The second line of evidence implicating endogenous DAG in the acute secretory action of TRH came from experiments examining the rapid activation of protein kinase C by TRH (11). We measured activation of protein kinase C by determining its subcellular localization at early times after treating GH$_4$C$_1$ cells with TRH (11). There was a rapid (< 10 sec) loss of protein kinase C activity or phorbol ester binding from the cytosol and also an equally rapid increase in protein kinase C in the particulate membrane fraction of the cell. Thus, it was possible to mimic TRH actions on $[Ca^{2+}]_i$ and secretion with ionomycin plus TPA and demonstrate that TRH activates rapidly protein kinase C, an important intracellular target for the action of endogenous DAG.

Is Ins(1,4,5)P₃ the Sole Mediator of Intracellular Calcium Redistribution in GH-Cells?

Results from several other investigators have shown that TRH activates phospholipase C and causes generation of Ins(1,4,5)P$_3$ in GH-cells within 2 to 5 sec. Ins(1,4,5)P$_3$ also causes calcium release from sequestered stores when added to permeabilized GH-cells. However, as indicated above, TRH causes an elevation of $[Ca^{2+}]_i$ in GH-cells within 300 msec (8). If Ins(1,4,5)P$_3$ that is generated in response to TRH is the intracellular mediator of TRH action on $[Ca^{2+}]_i$, it would be expected that enhanced Ins(1,4,5)P$_3$ formation would occur as rapidly, if not more rapidly, than the increase in $[Ca^{2+}]_i$ induced by TRH. When we examined the kinetics of Ins(1,4,5)P$_3$ in detail in GH$_4$C$_1$ cells, we found a lag of 1000 to 1200 msec before the increase in Ins(1,4,5)P$_3$ could be detected, while the rise in $[Ca^{2+}]_i$ occurred within about 300 msec (12). This result was not due to our inability to measure rapid changes in inositol polyphosphates,

because we could measure changes in InsP$_4$ and InsP$_5$ between 400 and 1000 msec (12,13). These findings suggest that the current dogma that Ins(1,4,5)P$_3$ is the sole mediator of intracellular calcium redistribution in GH-cells may need to be reconsidered.

Additional Aspects of Signal Transduction in GH-Cells

In the course of further studies with ionomycin, C.W. Fearon noted that low concentrations of the ionophore itself did not cause redistribution of protein kinase C, but that pretreatment with ionomycin antagonized the action of TRH (14). The antagonism required pretreatment for greater than 10 sec and was reversed by high K$^+$ in the presence of extracellular calcium. These results lead us to conclude that optimal activation of protein kinase C by TRH requires a simultaneous rise in both DAG and $[Ca^{2+}]_i$ (14).

GH$_4$C$_1$ cells were loaded with Quin 2 and the fluorescent proton reporter bis(carboxyethyl)carboxy-fluorescein (BCECF) and TRH-induced changes in pH$_i$ monitored (15). In acid loaded cells, TRH causes a rapid increase in pH$_i$ that is independent of changes in $[Ca^{2+}]_i$ and is blocked by amiloride. Thus, TRH stimulates the activity in the Na$^+$/H$^+$ antiporter in GH-cells. The mechanism appears to involve activation of protein kinase C, but this may not be the sole mode of control of pH$_i$ by TRH.

The acute action of TRH on $[Ca^{2+}]_i$ is modulated in GH$_4$C$_1$ cells by 1,25(OH)$_2$D$_3$. It has been shown that 1,25(OH)$_2$D$_3$ causes an increase in PRL synthesis and PRL mRNA accumulation in GH$_4$C$_i$ cells in a calcium-dependent manner (16,17). 1,25(OH)$_2$D$_3$ does not cause rapid (minutes) changes in $[Ca^{2+}]_i$. However, pretreatment of cells depleted of vitamin D with 1,25(OH)$_2$D$_3$ (10^{-10} M) for 24 to 48 hr causes a 2-fold enhancement of the TRH-induced spike in $[Ca^{2+}]_i$ with no change in the TRH-induced plateau phase of change in $[Ca^{2+}]_i$ (18). The mechanism does not involve a change in TRH receptors, in inositol phosphate generation and in protein kinase C activity, or an increase in the intracellular reservoir of calcium on which TRH acts. Because acute removal of extracellular calcium blocks the 1,25(OH)$_2$D$_3$ effect, the action of vitamin D appears to be mediated by enhanced influx of extracellular calcium induced by TRH (18). The TRH-regulated influx channel or pathway regulated by 1,25(OH)$_2$D$_3$ has not yet been identified.

Finally, we have solubilized the TRH receptor from GH-cells using 1% digitonin (19). Solubilization of the unoccupied receptor gives a soluble receptor that preserves the binding characteristics of the membrane or whole cell receptor including competition for TRH binding by chlordiazepoxide. Solubilization of the TRH-occupied receptor gives a soluble and functional TRH-receptor-G protein complex.

Conclusion

GH-cells have proven useful in identifying and characterizing a variety of important receptor-mediated transduction and regulatory pathways in protein hormone secreting cells. Although much has been learned, much more remains to be discovered using this novel cell culture system.

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